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3-Methylcholanthrene, Which Binds to the Arylhydrocarbon Receptor, Inhibits Proliferation and Differentiation of Osteoblasts *in Vitro* and Ossification *in Vivo*

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3-Methylcholanthrene (3MC) is a ligand for arylhydrocarbon receptor (AhR), which binds dioxin. We examined the effects of 3MC on the proliferation and differentiation of osteoblasts using cultures of rat calvarial osteoblast-like cells (ROB cells) and mouse calvarial clonal preosteoblastic cells (MC3T3-E1 cells). Analysis by RT-PCR revealed that the mRNAs for AhR and AhR nuclear translocators were expressed in both ROB and MC3T3-E1 cells. Cell proliferation and the synthesis of DNA by ROB cells and MC3T3-E1 cells were markedly inhibited on exposure of cells to 3MC. Furthermore, 3MC reduced the activity of alkaline phosphatase and the rate of deposition of calcium by cells. The level of expression of mRNA for osteocalcin, which is a marker of osteoblastic differentiation, was also depressed by 3MC. Moreover, when 3MC (1 mg/kg body weight) was administered sc to pregnant mice at 10.5, 12.5, and 14.5 d post coitus, fetuses examined subsequently at 15.5 or 17.5 d post coitus revealed evidence of inhibition of appropriate calcification of bones. The treated metacarpals showed no subperiosteal bone matrix histologically. Our findings indicate that 3MC might have critical effects on the formation of bone both *in vivo* and *in vitro*. (*Endocrinology* 143: 3575–3581, 2002)

3-METHYLCHOLANTHRENE (3MC) IS an aromatic hydrocarbon that is well known as a potent carcinogen (1, 2) and an inducer of the expression of a subclass of cytochrome P450 in liver (3). This compound also binds to the arylhydrocarbon receptor (AhR) as does dioxin (4). It has been proposed that 3MC and dioxin bind to the cytosolic AhR and then the receptor-ligand complex, together with an AhR nuclear translocator (Arnt), is relocated to the nucleus, with subsequent sequence-specific interaction with the xenobiotic-responsive element (XRE) of the arylhydrocarbon locus that results in the expression of multiple genes (5–7).

Osteoblasts are bone-forming cells. The formation of bone involves a complex series of events that include the proliferation and differentiation of osteoprogenitor cells and result eventually in the formation of a mineralized extracellular matrix. Several model systems have been developed for studies of the proliferation and differentiation of bone-forming cells *in vitro* and the molecular biology of the mineralization process (8–12). The sequential expression of type I collagen, alkaline phosphatase (ALPase) and osteocalcin, and the deposition of calcium are known as markers of osteoblastic differentiation. It has been suggested that environmental chemicals that disrupt endocrine systems might also affect the modeling and remodeling of bone. Gierthy *et al.* (13) provided evidence that 2,3,7,8-tetrachlorodibenzo-P-dioxin (TCDD), which is biologically the most potent known form of dioxin, inhibits the differentiation of osteoblast-like cells from fetal rat calvariae. Singh *et al.* (14) reported that an antagonist of the AhR, resveratrol, prevents the damaging effects of TCDD on bone formation. However, the effects and mechanisms of action of 3MC and dioxin on bone metabolism are still poorly understood. The potent toxicity of TCDD hinders experiments with this compound. Therefore, we examined the effects of 3MC, rather than those of TCDD, on the proliferation and differentiation of osteoblasts *in vitro* and on the mineralization by osteoblastic cells of fetuses in pregnant mice. Our results indicate that 3MC decreases biochemical indices of osteoblastic proliferation and differentiation and induces major alterations in the development and organization of bone tissue via its interactions with the AhR.

Abbreviations: AhR, Arylhydrocarbon receptor; ALPase, alkaline phosphatase; Arnt, AhR nuclear translocator; Brdu, 5-bromo-2′-deoxyuridine; dpc, days post coitus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KOH, potassium hydroxide; 3MC, 3-methylcholanthrene; MC3T3-E1, mouse calvarial clonal preosteoblastic cells; ROB, rat calvarial osteoblast-like cells; TCDD, 2,3,7,8-tetrachlorodibenzo-P-dioxin; XRE, xenobiotic responsive element.

Materials and Methods

Materials

3MC and staurosporine were purchased from Sigma (St. Louis, MO). ^{32}P-Labeled nucleotides were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). α-MEM, penicillin/streptomycin antibiotic mixture, and fetal bovine serum were obtained from Promega Corp. (Grand Island, NY).

Culture of osteoblastic cells

Rat calvarial osteoblast-like (ROB) cells were isolated enzymatically from calvariae of newborn Wistar rats as described previously (8). Mouse calvarial clonal preosteoblastic (MC3T3-E1) cells were supplied

by the RIKEN Cell Bank (Tsukuba, Japan). Cells were maintained in 55-cm^2 dishes in α-MEM, supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin, in a humidified atmosphere of 5% CO_2 in air at 37 C. After cells had reached 70% confluence, cells were detached by treatment with 0.05% trypsin, replated in either 55-cm^2 dishes or 12-well plates (area of each well, 3.8 cm^2) at a density of 1×10^4 cells/cm^2 and grown in α-MEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and 3MC at various concentrations. Fresh medium and 3MC were supplied to cells at 3-d intervals. ROB cells and MC3T3-E1 cells exhibited no changes in morphology during exposure to 3MC at concentrations from 10^{-9} M to 10^{-6} M. Cells detached by treatment with 0.05% trypsin were counted under a microscope (IX70, Olympus Corp., Tokyo, Japan).

RT-PCR

We detected mRNAs for mouse and rat AhR and Arnt in osteoblasts by RT-PCR. RNA was extracted from osteoblasts by the acid guanidinium-phenol-chloroform method (15). Total RNA (2 μg) was reverse transcribed by Moloney murine leukemia virus reverse transcriptase, SuperScript (200 U, Promega Corp.), with random primers (50 ng) in a 25-μl reaction mixture. The cDNA was amplified in 20 μl Taq DNA polymerase mixture (Takara, Tokyo, Japan) that contained 1 μM sense primer, 5′-GGGATTGATTTTGAAGATATCAG-3′, and antisense primer, 5′-AATGCCTGAGAACCTGGAATTTC-3′, for mouse AhR (accession no. D38417; 2536–3317, 782 bp); 1 μM sense primer, 5′-GGGATCGATTTC-GAAGACATCAG-3′, and antisense primer, 5′-AACGCCTGGGAGCCT-GGAATCTC-3′, for rat AhR (accession no. NM013149; 1657–2444, 788 bp); 1 μM sense primer, 5′-GCT ATA ATCATTCCCAGGTTTCT-3′, and antisense primer, 5′-CATTGTTGTAGGTGTTGCTTTGG-3′, for rat Arnt (accession no. NM012780; 1603–2367, 765 bp); 1 μM sense primer, 5′-GCTATAATCATTCCCAGGTTTCT-3′, and antisense primer, 5′-CAT-TGTTGTAGGTGTTGCTTTGG-3′, for mouse Arnt (accession no. NM009709; 1505–2272, 768 bp); or 1 μM sense primer, 5′-ACTTTGT-CAAGCTCATTTCC-3′, and antisense primer, 5′-TGCAGCGAACTT-TATTGATG-3′, for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (accession no. M32599; 957-1223, 267 bp). Each reaction cycle, performed 35 times for amplification of the cDNA consisted of incubation at 94 C for 30 sec, 55 C (rat AhR), 51 C (mouse AhR), and 60 C (rat and mouse Arnt and GAPDH) for 30 sec, and 72 C for 60 sec. Products of PCR were subjected to electrophoresis on a 2% agarose gel and visualized by staining with ethidium bromide. DNA marker fragments (Molecular weight marker V, Roche Molecular Biochemicals, Mannheim, Germany) were used as size markers.

Uptake of 5-bromo-2′-deoxyuridine (BrdU)

Osteoblastic cells were seeded in 96-well plates at a density of 3000 cells/well and subcultured for 36 h in α-MEM plus 10% fetal bovine serum, with and without 3MC at various concentrations. Subsequently, cells were incubated with BrdU for 12 h, and then cell proliferation was examined by enzyme immunoassay (5-bromo-2′-deoxy-uridine labeling and detection kit, Roche Molecular Biochemicals).

Measurement of alkaline phosphatase activity

Osteoblastic cells were subcultured in 12-well plates (3.8 cm^2/well) in α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and 3MC at various concentrations. The cells were washed with 10 mM Tris-HCl, pH 7.2, and sonicated in 1 ml of 50 mM Tris-HCl, pH 7.2, that contained 0.1% Triton X-100 and 2 mM $MgCl_2$ for 15 sec with a sonicator (ultrasonic disruptor UD-201, Tomy Co., Tokyo, Japan). The alkaline phosphatase activity of the sonicate was determined by an established technique with *p*-nitrophenyl phosphate as the substrate (8). Concentrations of protein were determined with BCA protein assay reagent (Pierce Chemical Co., Rockford, IL) with BSA as the standard.

Quantitation of the deposition of calcium

Osteoblastic cells were subcultured in α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and 3MC at various concentrations. The amount of calcium, in hydroxyapatite, in cell layers was measured as follows. The layers of cells in 12-well plates (3.8 cm^2/well) were washed with PBS, pH 7.4; PBS; 20 mM sodium phosphate and 130 mM NaCl) and incubated with 1 ml of 2 N HCl overnight with gentle shaking. The Ca^{2+} ions in samples were then quantitated, as described elsewhere (8), by the *o*-cresolphthalein complexone method with a calcium C kit (Wako Pure Chemical Industries, Osaka, Japan).

Northern blotting analysis

RNA was extracted from cells by the acid guanidinium-phenol-chloroform method (15). Total RNA (20 μg) was subjected to electrophoresis on a 1% agarose gel that contained 2.2 M formaldehyde and was then transferred to a MagnaGraph nylon membrane (Micron Separations Inc., Westborough, MA). After the membrane had been baked at 80 C for 2 h, the RNA on the membrane was allowed to hybridize overnight with cDNA for rat osteocalcin or rat GAPDH at 42 C in 50% formamide that contained 6× sodium chloride/sodium phosphate/EDTA solution (1× is 0.15 M NaCl; 15 mM NaH_2PO_4, pH 7.0; 1 mM EDTA), 2× Denhardt's solution (0.1% each of BSA, polyvinylpyrrolidone, and Ficoll), 1% sodium dodecyl sulfate, and 100 μg/ml herring sperm DNA. Each cDNA probe was radiolabeled with a Ready-to-Go kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The membrane was washed twice in 1× sodium chloride/sodium citrate (0.15 M NaCl, 15 mM sodium citrate, pH 7.0) that contained 0.1% sodium dodecyl sulfate at room temperature for 5 min each and twice in 1× sodium chloride/sodium citrate that contained 0.1% sodium dodecyl sulfate at 55 C for 1 h each and then it was exposed overnight to an imaging plate. The signals on the plate were analyzed with a Bioimage analyzer (BAS 2000, Fuji Photo Film Co., Ltd., Tokyo, Japan).

Preparation of animals

BALB/c female mice (9 wk old) were examined the morning after mating for the presence of vaginal plugs. Noon on the day of evidence of a vaginal plug was considered as 0.5 d post coitus (dpc). Pregnant mice were injected sc with 1 mg 3MC/kg body weight, dissolved in mineral oil, at 10.5, 12.5, and 14.5 dpc (*i.e.* three injections per mouse). Control dams received the solvent only. Fetuses at 15.5 or 17.5 dpc were obtained by cesarean section under anesthesia with diethyl ether. The fetuses collected from each pregnant dam were divided equally into two groups and used for whole-mount skeletal and histological examination, respectively. The animal protocols and procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Women's Medical University.

Analysis of fetal skeletons

For whole-mount skeletal analysis, fetuses at 15.5 or 17.5 dpc were placed in water for a couple of days, skinned, eviscerated, fixed in 95%

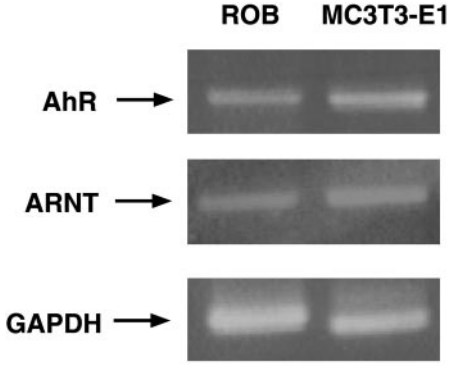

FIG. 1. Detection of transcripts specific for AhR and Arnt in ROB and MC3T3-E1 cells by RT-PCR. Total RNA was isolated from ROB and MC3T3-E1 cells and levels of mRNAs for rat AhR (788 bp), mouse AhR (782 bp), rat Arnt (765 bp), and mouse Arnt (768 bp) in the respective cells were examined by RT-PCR with specific primers (see text for details). Amplification of GAPDH mRNA was used as an internal control.

ethanol for 3 d, and finally stained with Alcian blue for 24 h. Skeletons were rinsed and dehydrated in 95% ethanol for 2 d and cleared in 1% potassium hydroxide (KOH) for 3–4 h. Samples were stained in alizarin red for 3–4 h and then rinsed in 2% KOH. They were cleared in 0.8% KOH plus 20% glycerol, 0.5% KOH plus 50% glycerol, and 0.2% KOH plus 80% glycerol and stored in 100% glycerol (16). For histological analysis, fetuses were fixed in 4% paraformaldehyde and then dehydrated through graded alcohols and embedded in paraffin. Paraffin blocks were sectioned at 6 μm thick and stained with hematoxylin-eosin.

Results

3MC binds to AhR in the cytosol and then the receptor-ligand complex, together with Arnt, is located to the nucleus. We performed an analysis by RT-PCR, using specific primers for amplification of rat and mouse genes for AhR and Arnt, as part of our efforts to characterize the effects of 3MC in osteoblastic cells. Figure 1 shows that we were able to detect transcripts specific for AhR and Arnt in both ROB and MC3T3-E1 cells.

When we examined the effects of 3MC on the proliferation of osteoblastic cells, we found that it inhibited increases in cell number (Fig. 2) and the synthesis of DNA (Fig. 3) by osteoblastic cells and that the effects were dose dependent. The exposure of cultures of both types of osteoblastic cell to 10^{-7} M 3MC inhibited increases in cell number after cells had become confluent. Exposure of cells to 3MC did not affect cell morphology, although cells became slightly hypertrophic. As shown in Fig. 3, 10^{-6} M, 3MC significantly inhibited the uptake of BrdU by ROB and by MC3T3-E1 cells. We confirmed, using Hoechst 33342 (Hoechst, Tokyo, Japan), that the effects of 3MC were not attributable to the induction of apoptosis of osteoblastic cells under our conditions of culture. The nuclei of osteoblastic cells that had been treated with 10^{-6} M 3MC for 72 h were similar to those of control cells (Fig. 4). By contrast, 10 μM staurosporine-treated cells (positive control) showed the condensation of nuclei.

3MC inhibited the differentiation of osteoblastic cells after confluence of cells. We exposed confluent osteoblastic cells to 3MC at various concentrations. Figure 5 showed that 3MC decreased the activity of ALPase (A) and the deposition of calcium (B) in both ROB and MC3T3-E1 cells. The ALPase activity in ROB and MC3T3-E1 cells that had been exposed to 10^{-7} M 3MC was significantly lower than that in control cells (treated with dimethylsulfoxide) after 9 and 12 d, respectively. The deposition of calcium by ROB and MC3T3-E1 cells was also strongly suppressed (to 10% and 50% of control values, respectively) by 3MC at 10^{-6} M. In ROB cells, the inhibition of the deposition of calcium was observed at 10^{-8} M 3MC. Northern blotting analysis revealed that 3MC inhibited the expression of mRNA for osteocalcin, which is a

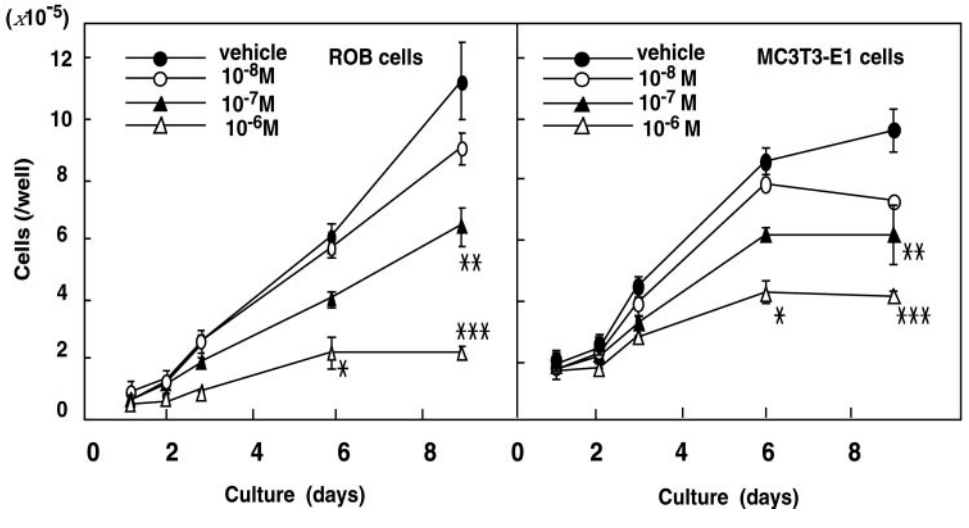

FIG. 2. Effects of 3MC on the proliferation of osteoblasts. ROB and MC3T3-E1 cells were cultured in 12-well plates (3.8 cm^2/well) with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and 3MC at various concentrations. Fresh medium with 3MC was supplied at 3-d intervals. At the times indicated, cells were removed from plates with trypsin solution and counted under a microscope. Values represent the means ± SD of results from three wells. Data are representative of results from three separate experiments. *, $P < 0.05$ *vs.* controls; **, $P < 0.01$ *vs.* controls; ***, $P < 0.001$.

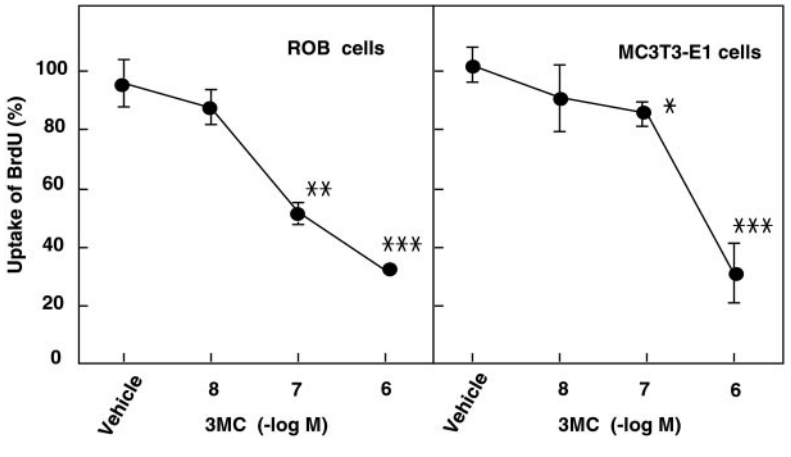

FIG. 3. Uptake of BrdU by osteoblasts exposed to 3MC. ROB and MC3T3-E1 cells were grown to confluence in 96-well plates. After growth of cells had been arrested by culture in serum-free medium for 72 h, cells were exposed to 3MC at various concentrations for 24 h. BrdU incorporated into osteoblasts was quantitated as described in the text. Values represent the means ± SD of results from four wells. Data are representative of results from three separate experiments. *, $P < 0.05$ *vs.* controls; **, $P < 0.01$ *vs.* controls; ***, $P < 0.001$ *vs.* control.

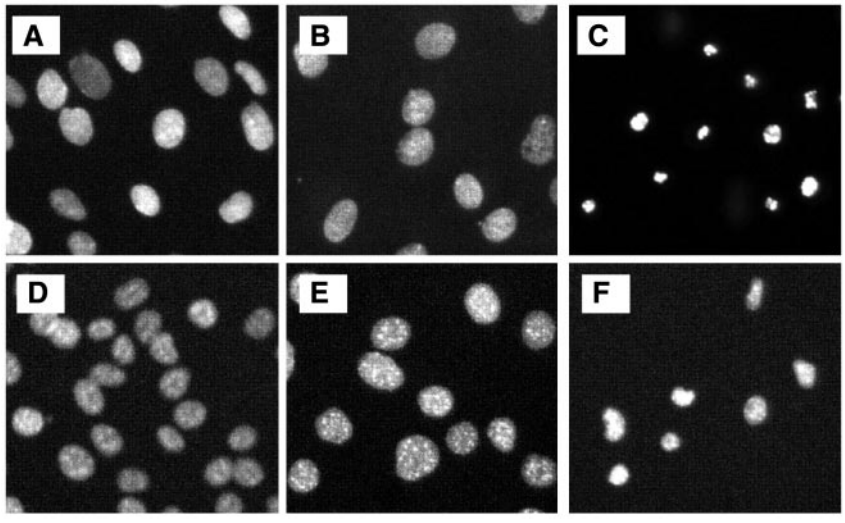

FIG. 4. The toxic effect of 3MC on osteoblastic cells. To visualized the nuclei of ROB cells (A–C) and MC3T3-E1 cells (D–F), the cells were stained with Hoechst 33342. The cells were treated with and without 10^{-6} M 3MC for 72 h and were then incubated with 0.2 μg/ml Hoechst 33342 for 15 min at 37 C. A and D, Control (0.1% dimethylsulfoxide); B and E, 10^{-6} M 3MC for 72 h; C and F, 10 μM staurosporine (positive control) for 2 h.

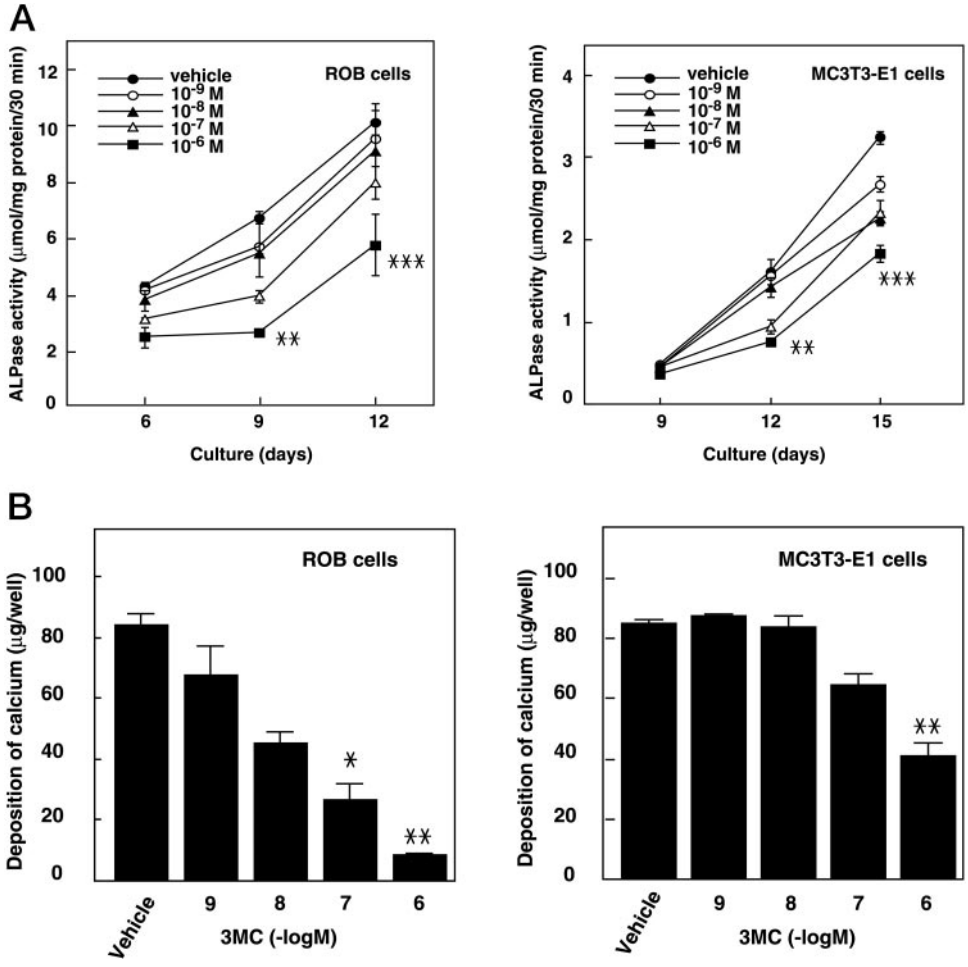

FIG. 5. Effects of 3MC on the ALPase activity (A) and mineralization (B) of ROB and MC3T3-E1 cells. ROB and MC3T3-E1 cells were cultured in 12-well plates (3.8 cm^2/well) with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid. 3MC at various concentrations was added to culture medium after confluence. Fresh medium with 3MC was supplied at 3-d intervals. At the times indicated, ALPase activity (A) and the deposition of calcium (at d 15 for ROB cells and at d 24 for MC3T3-E1 cells) (B) were measured as described in the text. Values represent the means ± SD of results from three wells. Data are representative of results from three separate experiments. *, $P < 0.05$ *vs.* controls; **, $P < 0.01$ *vs.* controls; ***, $P < 0.001$ *vs.* controls.

marker of osteoblastic differentiation (Fig. 6). On d 12, osteocalcin mRNA was expressed in control ROB cells, and its level of expression continued to increase with time. By contrast, low expression of osteocalcin mRNA was detected in ROB cells that had been treated with 10^{-7} M 3MC.

Because 3MC suppressed the proliferation and differentiation of osteoblastic cells in culture, we postulated that the signal caused by AhR might influence the early stages of growth and differentiation of embryonic or fetal bone. Therefore, we examined the effects of 3MC on the modeling of bone in fetuses in pregnant mice. We collected 58 fetuses that had been exposed to 3MC and 39 fetuses that had not been exposed to 3MC (controls) from 14 treated pregnant dams and 8 untreated pregnant dams, respectively. There were no dif-

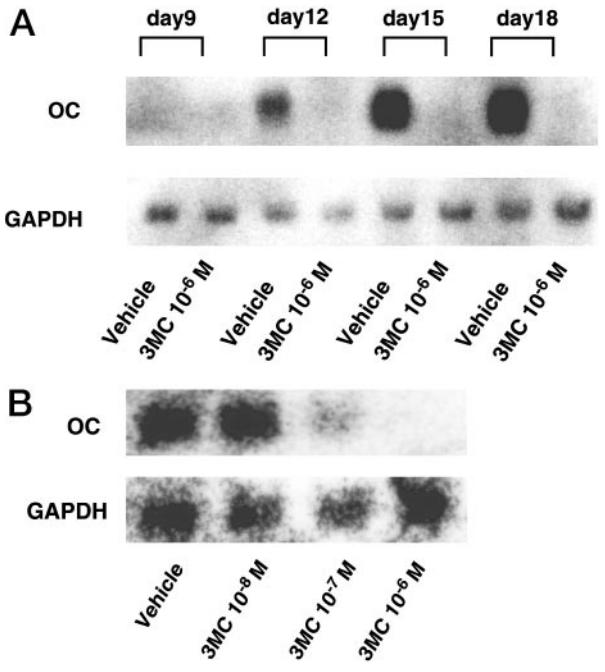

FIG. 6. Northern blotting analysis of mRNA for osteocalcin (OC) in ROB cells. Total RNA was isolated from cells after treatment with 10^{-6} M 3MC for 9, 12, 15, and 18 d (A) and treatment with various concentrations of 3MC for 15 d (B). Twenty micrograms total RNA were subjected to electrophoresis on an agarose gel and allowed to hybridize with ^{32}P-labeled cDNA for rat osteocalcin or GAPDH. Data represent results typical of those from three separate experiments.

ferences in body weight between fetuses from 3MC-treated and control dams. The fetuses collected from each pregnant dam were divided equally into two groups and used for whole-mount skeletal and histological examination, respectively. Whole-mount skeletal samples were prepared from 29 fetuses in the 3MC-treated group and 22 fetuses in the control group. The remaining fetuses were used for histopathological examination. Figure 7 shows typical photographs of fetal bones. We detected abnormalities in the first cervical (c1; *arrow* in Fig. 7B) and lumbar vertebrae in three fetuses in the 3MC-treated group and c1 in 3MC-treated fetuses was straight and tubelike in shape. We also noted a delay in the ossification of the cervical and thoracic vertebrae and limbs in nine fetuses in the 3MC-treated group, compared with the control group. In particular, ossification of the forelimb and hindlimb at 17.5 dpc was clearly delayed, as shown by *arrows* in Fig. 7, D and F, respectively. By contrast, abnormalities and a delay of the ossification were not observed in more proximal bones, such as ulna, radius, femur, and tibia.

Histologically, we compared metacarpals in the treated forelimb with control. The size and morphology of the chondrocytes in the proliferating and hypertrophic zones in the treated metacarpals were similar to those in control. Around the midsection of metacarpal 2, the pink-colored thin shell of the subperiosteal bone matrix was seen in control (*arrow* in Fig. 7I). In the treated metacarpal 2, however, no subperiosteal bone matrix was detected (compare Fig. 7, I with J). In the treated newborn animals, moreover, calcification in the primary marrow space and periosteum in the metacarpals was weak (data not shown). We detected calcification in the hypertrophic chondrocyte zone and vascular invasion in the primary ossification center.

Discussion

The specific risk to bone metabolism in mammals from exposure to environmental endocrine disruptors is not well understood. Among environmental endocrine disruptors, compounds that bind to AhR have the most potent toxic effects on animals. Therefore, we are attempting to clarify the potential effects of AhR ligands on bone metabolism. In the present study, we found that 3MC inhibited not only cell proliferation but also the differentiation of osteoblasts and bone formation *in vitro* using osteoblast model systems that had previously been well characterized. Furthermore, we showed that injection of 3MC, which binds to AhR, into pregnant dams affected the formation and ossification of bones during fetal development.

The AhR has been found in the cytosol of cells of almost all mammalian organs and tissues. We detected mRNAs for AhR and Arnt by RT-PCR in our osteoblast model systems, namely, ROB and MC3T3-E1 cells. Therefore, it seems possible that 3MC might affect bone formation through signals carried by AhR in osteoblasts. 3MC inhibited increases in cell number and the synthesis of DNA in ROB and MC3T3-E1 cells. Furthermore, after confluence of cells, 3MC inhibited the differentiation of ROB and MC3T3-E1 cells. 3MC reduced the levels of expression of markers characteristic of the osteoblast phenotype, namely ALPase and osteocalcin. Moreover, the deposition of calcium by ROB and MC3T3-E1 cells was inhibited by 3MC. These parameters are characteristic of the later stages of osteoblast differentiation and are essential for both the development and the maintenance of bone. Gierthy *et al.* (13) reported that TCDD at 10^{-8} M inhibited the differentiation of rat osteoblasts *in vitro*. 3MC at 10^{-7} M also decreased the proliferation and differentiation of rat and mouse osteoblasts. Our study confirms that AhR signals have a critical effect on the differentiation of osteoblasts. It seems to be differences in the effective doses of 3MC and TCDD on the differentiation of osteoblastic cells. However, there is a report that TCDD is much more potent than 3MC as an inducer of aryl hydrocarbon hydroxylase activity (5), although 3MC and TCDD bind to AhR with similar affinity (K_d = ~5 nM; 4).

The mechanism of the 3MC-induced alterations in the proliferation and differentiation of osteoblasts is unknown. The AhR-ligand complex is known to bind to the XRE (CACGCT/A) that is located in the 5′-flanking regions of target genes, and binding results in the expression of multiple genes (6, 7). However, there is no XRE in 5′-flanking regions of mouse genes for ALPase (accession no. X53659) (17) and osteocalcin (accession no. U66848) (18). Thus, it is likely that 3MC does not directly affect the expression of genes for markers of osteoblast differentiation. By contrast, Gierthy *et al.* (13) noted that there are similarities between the dioxin-induced suppression of osteoblast differentiation and the dioxin-mediated alterations in the responses to 17β-estradiol. Namely, TCDD inhibits estrogen-induced cathepsin D gene expression (19) and estrogen-induced responses in the rodent uterus (20) and mammary gland (21). Estrogen

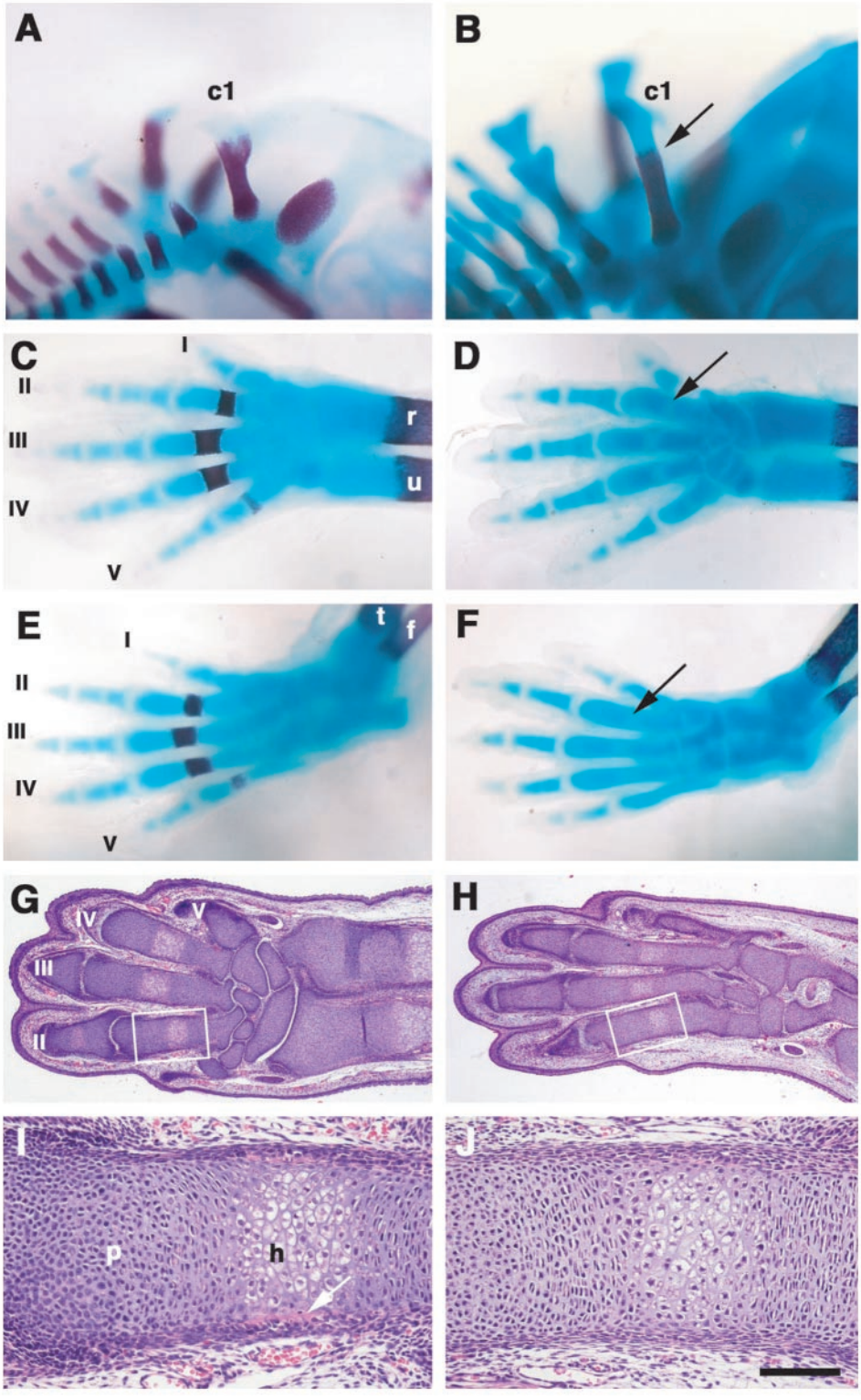

FIG. 7. The effects of 3MC on mouse fetuses. Analysis of whole-mount alizarin *red* and Alcian *blue*-stained (A–F) and histological sectioned (G–J) fetal skeletons. Bones of control (A, C, E, G, and I) and 3MC-treated (B, D, F, H, and J) fetuses are shown. A and B, Lateral views of the cervical vertebral region at 15.5 dpc. *Arrow* in B indicates delayed ossification of and morphological changes in the first cervical vertebra (c1). Bone stains *red* and cartilage stains *blue*. C and D, Dorsal views of bones of left forelimb at 17.5 dpc. *Arrow* in D indicates delayed ossification of the metacarpals. I–V, First to fifth digits; r, radius; u, ulna. E and F, Dorsal views of bones of left hindlimb at 17.5 dpc. *Arrow* in F indicates delayed ossification of the metatarsals. f, Fibula; t, tibia. G–J, Histological sections of the right forelimb at 17.5 dpc. Hematoxylin-eosin staining. I and J, Higher magnification of the second metacarpal of the specimens indicated in G and H, respectively. Note the *pink-colored* thin shell of the subperiosteal bone matrix *(arrow)* in I. h, Hypertrophic chondrocyte zone; p, proliferating chondrocyte zone. *Scale bar* in J, 100 μm.

is well known to stimulate the differentiation of osteoblastic cells. Therefore, the action of 3MC on osteoblastic cells might be, in part, through the estrogen-dependent pathways.

In our study, pregnant mice received injections of 1 mg 3MC/kg body weight at 10.5, 12.5, and 14.5 dpc during development. Injections over 1 mg 3MC/kg body weight were mortal for fetuses in dams. AhR mRNA is expressed in various primodia and organs of mouse embryos at 11.5 dpc (22). Therefore, we determined the treatment schedule of 3MC above. A delay of ossification was seen in the sternebrae, limbs, cervical and thoracic vertebrae, and supraoccipital bone in the 3MC-treated group, compared with the con-

trol group. We also observed morphological abnormalities of the first cervical and lumber vertebrae in the 3MC-treated fetuses. Delayed ossification was seen in the metacarpals and metatarsals in the treated limbs, although ossification of the metacarpals and metatarsals 2 to 5 in the limbs usually commences at 17.5 dpc (23). By contrast, the delay of ossification was not seen in more proximal bones, such as ulna, radius, femur, and tibia. The ossification of ulna and radius and femur and tibia commence at 14.5 dpc and 15.5 dpc, respectively (23). Therefore, we consider that the delay of ossification by 3MC is due to the timing of administration of 3MC.

We compared metacarpals in the treated and control fetuses histologically. Bone formation starts in the collar surrounding the hypertrophic cartilage core (24). In control metacarpals, the pink-colored thin shell of bone matrix that was due to substantial collagen content was seen under the periosteum. In treated metacarpals, however, no pink color of subperiosteal bone matrix and no clear osteoblasts were found. The proliferating and hypertrophic chondrocyte zones in metacarpals were not different from control. The hypertrophic cartilage core is eventually invaded by blood vessels and replaced by bone tissue and bone marrow. In treated newborn animals calcification in the primary marrow space and periosteum in the metacarpals was weak, compared with control (data not shown). A further detailed immunohistochemical study will give new insight into the mechanisms of 3MC.

The observations *in vivo* demonstrate that signals caused by AhR affect the modeling of bone in mouse fetuses. In rats, it has been reported that 3MC reduces retinoic acid-induced tail anomalies during fetal development (25). However, in our experiments, no tail anomalies were found in any fetuses. Silkworth *et al.* (26) examined the effects of a crude extract (containing TCDD at 170 ppb) of surface soil and leachate from the Love Canal chemical dump site in Niagara Falls, New York, on the fetal development of rats. They reported delayed ossification but no major treatment-related skeletal malformations in fetal rats. However, it remains important to perform experiments with pure TCDD. Our results *in vivo* and *in vitro* show that 3MC might affect bone metabolism in mice and rats. The effects mediated by interactions of AhR with 3MC or dioxin might result in bone abnormalities in humans also.

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